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Expression of *cry* genes from *Bacillus thuringiensis:* Influence on biochemical composition of transgenic cotton

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Continuing need to increase agricultural production has spurred the urge for developing cultivars with high productivity and a high degree of resistance to insect pests. One of the factors constraining crop production is losses due to insect pests, estimated at 14% of the total agricultural production (US$ 250 billion), despite application of insecticides valued at $50 billion annually (Oerke 2006). However, many insect species, including cotton bollworm, *Helicoverpa armigera* (Hubner), have developed high levels of resistance to conventional insecticides. Therefore, there is a need to harness all the technologies, including biotechnology for crop protection for a sustainable growth in agriculture and food security. To achieve a satisfactory control of insect pests, genes encoding d-endotoxins from *Bacillus thuringiensis* (*Bt*) have been deployed in a number of crops including cotton, and approved for commercial cultivation in several countries. Because of the potential benefits of growing genetically modified crops, their cultivation has increased from 1.97 million ha in 1996 to over 160 million ha in 2011 (James 2011). Cotton cultivars with *Bt* genes for resistance to cotton bollworm, *H. armigera* have resulted in a significant decrease in number of insecticide sprays applied for bollworm control in cotton, and increased cottonseed yield (Sharma *et al*. 2004, Sharma and Pampapathy 2006, Dhillon *et al.* 2012). Although, the promise of genetically modified crops has been realized in several crops and in different regions for increasing crop production, it is important to address the concerns related to their impact on non-target organisms, biochemical composition and substantial equivalence to the conventional food.

Plants are known to produce a diverse array of biochemical constituents in different quantities and proportions, which affect the behavior and biology of phytophagous insects. One of the concerns of biosafety of genetic engineering is the changes in biochemical composition of the plants, including the secondary metabolites, as a result of insertion and expression of exotic genes. Tannins and polyphenols, which are widespread in plants, are considered to be an essential component of plant defense system against environmental stresses, including insect herbivores. The chemical basis for the defensive role of tannins has been attributed to their ability to precipitate plant proteins and inhibit gastrointestinal enzymes, thereby, reducing the digestibility of plant proteins (Zucker 1983). Polyphenols constitute one of the most common and widespread groups of substances in plants, have no specific metabolic function in plant cells, but are essential for providing defense against biotic stresses (Bennick 2002).

The ideal transgenic plant is expected to have a single intact copy of the desired transgene inserted into a non-functional region of the plant genome, without further alteration of the host plant DNA, which cannot be pre-selected and targeted to non-functional regions of the genome (Puchta 2003). However, it is possible to select insertion events that consist of a single intact t-DNA with no known function, and is free from deletions and rearrangements because of insertion of superfluous DNA, if sufficient numbers of transgenic events are generated (Wilson *et al.* 2006). There is no complete sequencing of the deregulated t-DNA insertion events in cotton, and there is a possibility of getting transformation-induced mutants from the transgenic plants. To test for unintended positional and pleiotropic effects, the methylation patterns of the genes in the flanking host genome DNA and total protein and metabolic profiles must be characterized in *Bt*-transformed and the non-transformed plants of the same genotype. Studies on protein profiles would help to monitor the unintended changes in the pattern of gene expression, while the metabolic profile would help in monitoring the unintended changes in metabolism and the

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amounts of secondary metabolites. Therefore, we studied the effects of genetic transformation of cotton with *Bt* genes on the nutritional composition and amounts of secondary metabolites in different plant parts of transgenic and non-transgenic cottons.

Studies were conducted on *Bt*-transgenic cotton hybrids Mech 184 expressing *cry1Ac* gene and MRC 7201 BGII with *cry1Ac* + *cry2Ab* stacked genes, and their non-transgenic counterparts (Monsanto Mahyco Pvt Ltd, India). The plants were grown under stress free environment (biotic and abiotic) in the greenhouse at the International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India. Each test genotype was grown in 15 pots (28 cm in depth × 32 cm dia.) filled with a potting mixture of black soil and farmyard manure (3: 1) + 20 g diammonium phosphate as a basal fertilizer. There were five pots for each genotype and each pot had five plants. The leaves from 30 days old seedlings, 3 to 4 day-old squares, and 7 to 8 day old bolls were collected from the designated pots. The leaf petioles and sepals from squares and bolls were removed from the test samples. The samples were collected in plastic bags and immediately kept in an icebox, which were transferred into a deep freeze (–20°C). The plant samples from the deep freeze were lyophilized at –45ºC for 5 days. The lyophilized samples were powdered in a Willey mill, and used for the biochemical analysis.

The total protein content was estimated by AOAC (1995), total sugars by phenol - sulphuric acid method (Dubois *et al.* 1956), polyphenols by Folin Denis method (AOAC 1984), and tannins by vanillin - hydrochloric acid method (Price *et al.* 1978). Data were expressed in mg/g dry weight. The freeze-dried and powered leaf, square and boll samples of the test *Bt*-transgenic and the non-transgenic cotton hybrids were collected in individual Eppendorf tubes and crushed in a PBS buffer in the ratio of 1:10 (sample: buffer) with a plastic pastel. To estimate the amounts of Cry1Ac protein in the samples, semi-quantitative ELISA (Agdia®) was performed using the procedure of Sharma *et al.* (2008), along with *Bt* protein standards of 0.3125, 1.25, 5, 10, and 20 ppb of Cry1Ac. The data were subjected to analysis of variance in a factorial design to test the effects of genotype, *Bt*-transgene, plant parts, and their interaction effects on the amounts of various biochemical constituents, and the treatment means were compared by least significant differences (LSD) at *P*=0.05.

The leaves of genetically transformed cottons with *Bt*-genes had significantly ($F_{1,3}$ = 15.47; P = 0.003) higher amounts of total protein as compared to the non-transformed counterparts. Leaves of Mech 184 had greater amounts of proteins than in MRC 7201 BGII ($F_{1,3}$ = 13.23; P = 0.005). However, total protein content in squares and bolls of *Bt*-transgenic cottons was lower than in non-transgenic counterparts, but the differences were nonsignificant (Fig 1). The protein content was significantly ($F_{2,6}$ = 6.72; P = 0.003)

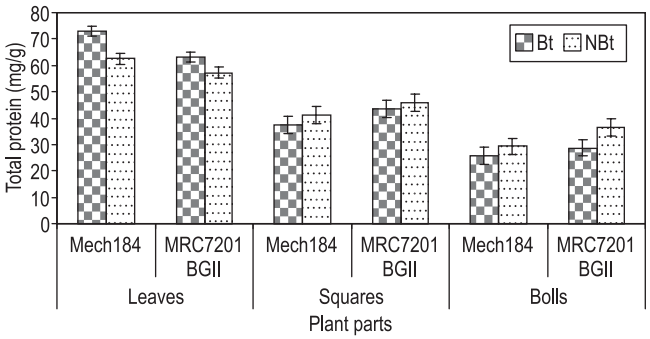

Fig 1 Total protein content in different plant parts of *Bt* and non-*Bt* cottons.

greater in leaves followed by squares and bolls (leaves > squares > bolls) of *Bt*-transgenic and non-transgenic cottons. Higher protein content in leaves than that in bolls has earlier been reported by Renuka *et al.* (2005). The differences in total sugar content in the leaves, squares and bolls of *Bt*-transgenic and nontransgenic cottons were nonsignificant. Earlier studies have reported lower sugar content in *Bt*-transgenic cotton than in the non-transgenic controls (Wu *et al.* 2000, Lu *et al.* 2005). There were significant differences in sugar content in different plant parts ($F_{2,6}$ = 539.17; P = <0.001) of both *Bt*-transgenic and non-transgenic cottons. The total sugar content was significantly higher in bolls, followed by squares and leaves.

The tannin content in the leaves, squares and bolls of *Bt*-transgenic cotton was significantly lower than that of the non-transgenic counterparts (Fig 2). There were significant differences in tannin content in different plant parts ($F_{2,6}$ = 5.71; P = 0.007) of the *Bt*-transgenic and non-transgenic cotton. The tannin content was significantly higher in bolls, followed by squares and leaves in both *Bt*-transgenic and non-transgenic cottons. The condensed tannins in terminal leaves of *Bt*-transgenic cottons have been reported to be significantly lower as compared to the non-transgenic controls (Wu *et al.* 2000, Lu *et al.* 2005). However, tannin content in terminal leaves increased with plant age (Wang *et al.* 1997).

The polyphenol content of leaves, squares and bolls of *Bt*-transgenic cotton was significantly lower than that of non-transgenic counterparts. However, there were significant differences in polyphenol content in leaves ($F_{1,3}$ = 12.29; P = 0.007), squares ($F_{1,3}$ = 9.74; P = 0.012) and bolls ($F_{1,3}$ = 28.97; P = <0.001) of *Bt*-transgenic and non-transgenic cottons. Earlier studies have reported lower amounts of total phenols in *Bt*-transgenic cotton as compared to the non-transgenic controls (Wu *et al.* 2000, Lu *et al.* 2005). Leaves and bolls of MRC 7201 BGII had significantly higher polyphenol content than that of Mech 184, while the reverse was true in case of squares. There were significant differences in polyphenol content of different plant parts ($F_{2,6}$ = 18.07; P = <0.001). The bolls and squares had significantly more polyphenols than the leaves in both *Bt* and non-*Bt* cottons.

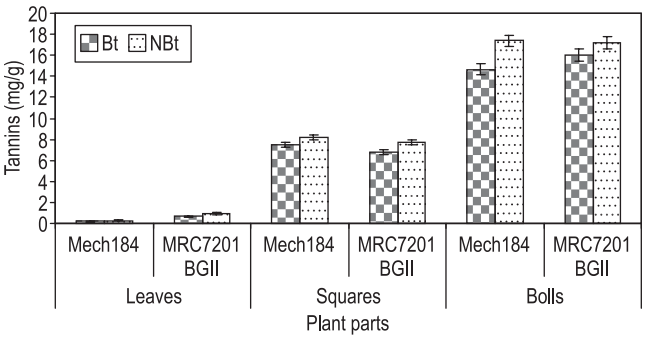

Fig 2 Production and accumulation of condensed tannins in different parts of *Bt* and non-*Bt* cottons.

The leaves of *Bt*-transgenic Mech 184 and MRC 7201 BGII contained 14.4 and 16.7 ng/g of *Bt* protein, respectively, while no *Bt* toxin was detected in the leaves of non-transgenic cotton. The leaves of *Bt*-transgenic cotton hybrid MRC 7201-BGII had greater amounts of Cry1Ac *Bt* toxin than in Mech 184. The *Bt* protein content was below detectable limits in squares and bolls of *Bt*-transgenic cotton during the flowering and fruiting stages. Chen *et al.* (2002) reported that the expression of *Bt* protein/toxin varies with the age and stage of the plant, which results in less effectiveness of *Bt* cottons for bollworm control during the later stages of plant growth.

Decrease in tannin content is associated with an increase in *Bt* toxin, and hence, resistance to cotton bollworm, *H. armigera* (Chen *et al.* 2002). Navon *et al.* (1993) suggested that cotton plants with high tannins may not be compatible with *Bt* used as a microbial pesticide or deployed in transgenic plants for pest management. One of the characteristic properties of tannins is their ability to precipitate proteins in aqueous solutions. Since most of the biological activity of tannins is believed to be related to their protein binding properties, but there is a reversible interaction between polyphenols and proteins in solution, leading to an equilibrium between the soluble protein/tannin complexes, and the reactants (Luck *et al.* 1994). Lower amounts of tannins and polyphenols in *Bt*-transgenic cotton might be because of insertion of the *Bt* genes at a site affecting the synthesis and accumulation of secondary metabolites or genetic differences between the *Bt*-transgenic and counterparts because of partial conversion of the non-transgenic genotype with the transgenic event used in developing the transgenic hybrids. There is a need to deploy *Bt* toxin genes in cultivars with lower amounts of tannins, and have stable expression of the protein during different stages of plant growth for effective control of the bollworms.

SUMMARY

The promise of genetically modified cotton has been realized in several parts of the world for insect control, however, the substantial equivalence of transgenic plants with the non-transgenic version of the same genotype in terms of nutritional quality and the amounts of secondary metabolites is of prime importance for ensuring the biosafety of transgenic plants for human beings and the environment. There were no major changes in protein and sugar contents, while the amounts of polyphenols and tannins were significantly lower in *Bt* than in non-*Bt* cottons. Amounts of condensed tannins and polyphenols were greater in bolls and squares than in leaves, while reverse was true in case of total proteins. Greater amounts of *Bt* toxin were detected in leaves, but were below detectable level in squares and bolls of *Bt* cotton, which possibly results in less effectiveness of *Bt* cotton for bollworm control during the later stages of plant growth.

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